Journal of Ethnopharmacology 256 (2020) 112706

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

The effects of hydro-ethanolic extract of *Capparis spinosa* (*C. spinosa*) on lipopolysaccharide (LPS)-induced inflammation and cognitive impairment: Evidence from *in vivo* and *in vitro* studies

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ARTICLE INFO

Keywords:
Capparis spinosa
Microglia
Cognitive impairment
M1/M2
LPS
Inflammation

ABSTRACT

Ethnopharmacological relevance: *Capparis spinose* (*C. spinosa*) belonging to Capparaeae, originates from dry areas in the west or central Asia and Mediterranean basin. For thousands of years, *C. spinosa* has been reported to be used as a therapeutic traditional medicine to relieve various ailments including rheumatism, pain and inflammatory diseases.

Aim of the study: There are several studies mentioning that systemic inflammation results in learning and memory impairments through the activation of microglia. The objective of this study was to investigate the effect of *C. spinosa* on both *in vivo* and *in vitro* models of neuroinflammation and cognitive impairment using lipopolysaccharide (LPS).

Materials and methods: In vivo: 40 male rats were used in the present study. Cognitive impairment was induced using LPS (1 mg/kg/d; i.p.) for 4 weeks. Treatment with *C. spinosa* (100 and 300 mg/kg/d; p.o.) was performed 1 h before LPS administration. At the end of the experiment, rats were undergone for behavioral and biochemical analysis. In vitro: Primary microglia isolated from mouse was used in the present study. The cells were pretreated with *C. spinosa* extract (10–300 μg/ml) and then stimulated with LPS (1 μg/ml). The expression levels of inflammatory and anti-inflammatory cytokines were elucidated using Real-Time PCR and ELISA methods.

Results: The escape latency in the Morris water maze test in the LPS group was significantly greater than the control group ($p < 0.001$), while, in extract-treated groups, it was less than the LPS group ($p < 0.001$). Additionally, we found that the levels of IL-1β, TNF-α, and iNOS/Arg-1 ratio was also significantly lower in extract-treated groups than the LPS group ($p < 0.001$). The results revealed that C. spinosa extract significantly reduced the levels of TNF-α, iNOS, COX-2, IL-1β, IL-6, NO and PGE2, and the ratios of iNOS/Arg-1 and NO/urea, following the LPS-induced inflammation in microglia ($p < 0.001$).

Conclusions: Our finding provides evidence that *C. spinosa* has a neuroprotective effect, and might be considered

Abbreviations: AD, Alzheimer's disease; Arg1, Arginase 1; DMSO, Dimethyl sulfoxide; MTT, (3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyl tetrazolium bromide; DMEM, Dulbecco's Modified Eagle's Media; ELISA, Enzyme-linked immunosorbent assay; FBS, Fetal bovine serum; iNOS, inducible nitric oxide synthase; IL-1β, interleukin; IL-4, interleukin; LPS, Lipopolysaccharide; TNF-α, tumor necrosis factor; NO, nitric oxide; PBMC, peripheral blood mononuclear cells; PGE_2, prostaglandin E_2

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https://doi.org/10.1016/j.jep.2020.112706
Received 26 September 2019; Received in revised form 20 February 2020; Accepted 21 February 2020
Available online 25 February 2020

as an effective therapeutic agent for the treatment of neurodegenerative diseases that are accompanied by microglial activation, such as AD.

1. Introduction

Neuroinflammation and its chronicity in the brain are closely associated with the pathogenesis of a variety of neurodegenerative diseases including Alzheimer's disease (AD), Parkinson's disease, Huntington's disease, multiple sclerosis (MS), ischemic stroke (Askari et al., 2018b; Askari and Shafiee-Nick, 2019a; Chen et al., 2016; Zipp and Aktas, 2006). AD is considered a well-known progressive neurodegenerative disease, which is often connected with cognitive impairment surprisingly in the elderly population. Recently, several studies have established that inflammation plays a pivotal role in the pathogenesis of AD. In this context, it has been indicated that systemic and local inflammation can impress the mental functions, including learning, memory and neuronal plasticity (Beheshti et al., 2019; Harrison et al., 2014). Furthermore, it has been illustrated that inflammation acts an important role during learning and memory impairments (Harrison et al., 2014). There is ample of evidence demonstrating that brain inflammation is tightly associated with the spatial learning impairment and memory dysfunction (Beheshti et al., 2019; Harrison et al., 2014; Kim et al., 2012). Additionally, it has been reported that the systemic inflammation is associated with cognitive impairment (Kim et al., 2012).

Microglial cells are the major immune cells that reside in the brain and play critical roles in neural homeostasis and neuroinflammation due to lake of adaptive immune system (Askari et al., 2018a; Askari and Shafiee-Nick, 2019a; Kaur et al., 2010; Kianmehr et al., 2017). The microglial cells exhibit a similar pattern to other macrophages, depending on the predominance of secreted factors, that two main phenotypes have been characterized for microglia, namely M_1 (inflammatory cells; producing higher levels of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, prostaglandin E_2 (PGE_2), nitric oxide (NO), and inducible nitric oxide synthase (iNOS)) and M_2 (anti-inflammatory cells; producing higher levels of IL-10, arginase 1 (Arg1)) (Orihuela et al., 2016). Several experimental studies have indicated that lipopolysaccharide (LPS) activates microglial cells through toll like receptor-4 (TLR-4) (He et al., 2018; Tai et al., 2018). Microglia activation by LPS appears to exacerbate AD pathology associated with tau phosphorylation (Lee et al., 2010), amyloidogenesis and memory impairment via TLR4 activation (He et al., 2018) as well as degenerative effect on the dopaminergic system (Bansal and Singh, 2018; Tai et al., 2018). In fact, several studies mentioned that both systemic and local exposure to LPS result in inflammation, learning and memory impairments through activation of microglia and increase in the levels of inflammatory cytokines and free radical production (Askari and Shafiee-Nick, 2019a; Beheshti et al., 2019; Harrison et al., 2014; Kim et al., 2012). Considering the important role of microglia-mediated neuroinflammation in the pathogenesis and progression of neurodegenerative diseases, alterations in microglia M_1/M_2 polarization could be considered an emerging strategy for the prevention and treatment of several neurodegenerative diseases, namely AD and MS (Hu et al., 2012; Liu et al., 2015).

Capparis spinose (*C. spinosa*), which belongs to Capparaeae, originates from dry areas in west or central Asia and Mediterranean basin (Trombetta et al., 2005). For thousands of years, *C. spinosa* has been reported to be used as therapeutic traditional medicine to relieve various ailments (Aliyazicioglu et al., 2013; Romeo et al., 2006) such as rheumatism, rheumatoid arthritis and gout (Rahnavard and Razavi, 2016; Zhang and Ma, 2018). In the traditional medicine, *C. spinosa* has been applied for treatment different types of pain since ancient times (Fu et al., 2008). Different parts of the medicinal plant (aerial parts, roots, and seeds) have been known to exhibit multiple biological activities including anti-inflammatory, anti-allergic and anti-histaminic, antiviral and immune-modulatory, antioxidant, antimicrobial properties (Kulisic-Bilusic et al., 2012; Tlili et al., 2011; Trombetta et al., 2005). Moutia et al., 2016 recently showed that aerial part of *C. spinosa* up-regulates the gene expression of an anti-inflammatory cytokine interleukin (IL)-4, whereas down-regulates the levels of pro-inflammatory cytokine IL-17 gene expression, in human on peripheral blood mononuclear cells (PBMC) (Zhang and Ma, 2018). Basis on this, in the present study, we aimed to evaluation of the anti-inflammatory and immunomodulatory effects of *C. spinosa* both *in vivo* and *in vitro* (microglia) against LPS-induced neuroinflammation and memory dysfunction.

[1] These authors share the first co-authorship equally.

2. Material and methods

2.1. Chemicals and reagents

Dulbecco's Modified Eagle's Media (DMEM)/F12 culture media, penicillin/streptomycin (pen/strep), amphotericin-B, Fetal bovine serum (FBS), Dimethyl sulfoxide (DMSO), Ficoll, DNAse I, Dispase II, Lipopolysaccharides (LPS), and other cell culture chemicals were provided from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Red blood cells (RBC) lysis buffer was obtained from Biolegend company (San Diego, CA, USA). (3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyl tetrazolium bromide (MTT) was from Roche Diagnostic (Mannheim, Germany). IL-6, IL-10, IL-β, TNF-α ELISA kits were purchased from and eBioscience (San Diego, CA, USA). All other materials were of analytical and standard grade and purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Urea kit was obtained from Abcam.

2.2. Plant collection and extraction

The aerial parts of *C. spinosa* were collected from plants cultivated in the Kalat region, Khorasan Razavi province, Iran. The identification of the specimen was established by a botanist (Mrs. Sozani) at School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran, and deposited in the herbarium of Ferdowsi University of Mashhad (herbarium No. 13063).

C. spinosa aerial parts were air dried in the shadow and ground to fine powder. The extraction was done by the maceration method. In brief, the dried powdered aerial parts (100 g) were soaked in 70% v/v of the hydro-ethanolic solution for 72 h along with light shaking. Afterward, the mixture was filtered using Whatman No. 1 filter paper. After centrifuging at 3000 rpm (round per minute) for 5 min, the supernatant was concentrated by a rotary evaporator (37 ˚C) and then were undergone freeze dry process to eliminate ethanol/water and prepare a fine powder. The yield of the extract was 18.9% w/w of the dry powder, and was stored at -80 ˚C until used.

2.3. Liquid chromatography–mass spectrometry (LC-MS) apparatus and standardization procedures

The LC-MS analysis was performed in an AB SCIEX QTRAP (Shimadzu) liquid chromatography equipped with triple quadrupole Mass Spectrometer. Liquid chromatography separation was performed on a Supelco C18 (15 mm × 2.1 mm × 3 μm) column. The analysis was done at a flow-rate of 0.2 ml/min with a mixture of methanol and water (90:10) and the mass spectra were acquired in a range of 150 to 1700 within the 60 min scan time. The positive electrospray ionization (ESI)

mode was applied for the Mass Spectrometer. Mass feature extraction of the acquired LC-MS data and maximum detection of peaks was done using MZmine analysis software package, version 2.3.

2.4. In vivo study

2.4.1. Animals and husbandry

Forty male Wistar rats (weighted 230 ± 15 g) were purchased from the animal laboratory of Mashhad University of Medical Sciences. Then, all rats were randomly divided into four groups of ten in separately ventilated cages (22 ± 2 °C, humidity of 54 ± 2% and 12 h light/dark cycle). All animals had free access to food and taped drinking water. The experimental procedures were approved by *the Ethical Committee of Animal Research* (961339; IR.MUMS.MEDICAL.REC.1397.041).

2.4.2. Study design and experimental groups

LPS was freshly prepared in sterile saline before injection. Grouping was performed as follows (Table 1); Group 1, Control: rats received saline instead of either LPS or the extract; Group 2, LPS: rats received daily and intraperitoneally (i.p.) injection of LPS (1 mg/kg) for 4 weeks and also 120 min before behavioral tests. These animals received 5 ml/kg saline instead of the extract.

Groups 3 and 4, the extract treated: rats received 100 mg/kg/d and 300 mg/kg/d of the extract by oral gavage, 1 h before LPS injection (1 mg/kg/d; i.p.); the rats were treated for 4 weeks. Selection of doses of the extract ((Goel et al., 2016; Kalantari et al., 2017; Turgut et al., 2015)) and LPS ((Beheshti et al., 2019)) were according to the previously published studies.

2.4.3. Morris water maze (MWM) test

The MWM test was carried out using the black circle pool (60 cm depth, and 150 cm diameter) half full of water (24 ± 1 °C), describing elsewhere (Beheshti et al., 2019). Briefly, the procedure included five consecutive days of training with another day for a probe test. In this experiment, the evaluations were performed four trials for each rat per day from different release positions to find the location of a plexiglas platform (located two cm below the water level). Noteworthy, if the rat was not able to find the platform during 60 s of the acquisition phase, it was climbed on the platform for 15 s. During the training days, the distances traveled and the time spent to find the platform were assessed and compared as behaviors between the groups. Probe test was done on the 6th day without the platform. The rat was recorded for 60 s to search the maze, and the time spent in the platform position was recorded. A video-tracking system was used to record the latency of finding the platform.

2.4.4. Measurement of the levels of TNF-α, IL-1β, and IL-10 by ELISA

The levels of TNF-α, IL-1β, and IL-10 were measured in the whole brain tissue using ELISA kits according to the manufacturer's protocol (Askari et al., 2018b, 2018c; Askari and Shafiee-Nick, 2019b; Lee and Tansey, 2013; Rahimi et al., 2018).

2.4.5. Quantitative real-time PCR (qPCR)

Whole brain tissue was homogenized and transferred into the TRIzol reagent for RNA extraction. RNA was extracted in accordance with the manufacturer's instruction. Primer sequences used for Arg-1 (Forward: 5′-GGCAGTGCCGTTGACCTTGT-3′, Reverse: 5-AGCAGCGTTGGCCTGGTTCT-3' (Amrouni et al., 2011)), iNOS (Forward: 5′-CACCACCCTCCTTGTTCAAC-3′, Reverse: 5′-CAATCCACAACTCGCTCCAA-3′ (Nergiz et al., 2012)) and Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH, Forward: 5′-GGAGAAAGCTGCTAA-3′, Reverse: 5′-ACGACCTGGTCCTCGGTGTA-3' (Sobajima et al., 2005)) were according to the previous studies designed with mRNA sequences. The real-time PCR reactions were performed using the SYBR® Green Master Mix and reference genes quantification by a Rotor-Gene Q Real-Time PCR machine (Corbett Research, Australia) according to our previous studies (Askari et al., 2016, 2018c; Askari and Shafiee-Nick, 2019b; Lee and Tansey, 2013; Rahimi et al., 2018). Statistical analysis of gene expression levels was performed by the $2^{-\triangle\triangle Ct}$ method and expressed as fold-changes of the control group.

2.5. In vitro study

2.5.1. Microglia isolation, cell culture and treatment

Primary microglial cells were isolated from 8-weeks-old (28 ± 5 g) male C57BL/6 mice as described in our previous studies (Askari et al., 2018c; Askari and Shafiee-Nick, 2019b; Lee and Tansey, 2013; Rahimi et al., 2018). The microglial cells isolated from adult mice brain were cultured in enriched-DMEM (1% v/v of Pen/Strep (100 x), 10% v/v of heat-inactivated FBS, 0.5 μg/mL amphotericin B, and 2 mM L-glutamine) in a humidified incubator at 37 °C, where CO_2 accounted for 5% v/v. The cultured cells were treated with 1 μg/ml LPS, the optimal concentration to stimulate the cells, in the presence or absence of 30–300 μg/mL *C. spinosa* extract or dexamethasone (0.5 μM) as positive control (Feng et al., 2017). Treatment groups were presented in Table 1. *C. spinosa* extract was dissolved in DMSO to prepare a concentration of 50 mg/mL and serially diluted with the complete medium. For all experiments, the level of DMSO was lower than 0.1% v/v, and the different concentrations of the extract freshly prepared.

2.5.2. Cell proliferation assay

Microglial cells were seeded at a density of (1×10^5 cells/ml) to 96-well plates. Afterward, the cells were treated with *C. spinosa* extract (1–300 μg/ml), dexamethasone (0.5 μM) as positive control (Feng et al., 2017), and *C. spinosa* extract (30–300 μg/ml) or dexamethasone (0.5 μM) as positive control + LPS (1 μg/ml), and incubated for 48 h at 37 °C in 5% CO_2 incubator. MTT solution (0.5 mg/ml) subsequently was added to each well which incubated for next 3 h. Then, the culture media were removed and replaced by DMSO (100 μl/well) to dissolved crystals. The absorbance was read at 570 nm in referencing 620 nm with a StatFAX 2100 microplate reader (Awareness Inc, USA) (Askari et al., 2018c; Askari and Shafiee-Nick, 2019b; Lee and Tansey, 2013; Rahimi et al., 2018).

2.5.3. Measurement of the levels of TNF-α, IL-1β, IL-6, and PGE_2

The levels of inflammatory mediators were assessed in the culture medium collected from microglial cells after treatment with LPS (1 μg/ml) or LPS with the extract or dexamethasone as positive control by ELISA kits according their instructions (Askari et al., 2018b, 2018c; Askari and Shafiee-Nick, 2019b; Lee and Tansey, 2013; Rahimi et al., 2018).

Table 1
Protocol of treatment groups.

In vivo study			
Groups	Vehicle	LPS (1 mg/kg/d)	*C. spinosa extract* (mg/kg/d)
1	√	–	-
2	√	√	-
3	√	√	100
4	√	√	300
***In vitro* study**			
Groups	Vehicle	LPS (1 μg/ml)	*C. spinosa* extract (μg/ml)
1	√	–	–
2	√	–	300
3	√	√	–
4–6	√	√	30, 100 and 300
7	√	√	0.5 μM Dexamethasone
8	√	–	0.5 μM Dexamethasone

2.5.4. Quantitative real-time PCR (qPCR)

Microglial cells were incubated with LPS (1 μg/mL) in the presence or absence of 10, 30, 300 μg/mL of *C. spinosa* extract or dexamethasone (0.5 μM) as positive control for 6 h. GAPDH as the reference gene, was used to evaluate the relative expression of iNOS, TNF-α, and IL-1β. Grouping was done according to the experimental design, while the duration of exposer to evaluate the mRNA for all groups was 6 h. Primer sequences used for Arg-1, iNOS, TNF-α, Il-1β, IL-6 has been depicted in Table 2. The primers sequences were obtained according to the previous studies designed with mRNA sequences (Askari et al., 2016, 2018c; Askari and Shafiee-Nick, 2019b; Lee and Tansey, 2013; Rahimi et al., 2018). The real-time PCR reactions were performed using the SYBR® Green Master Mix and reference genes quantification by a Rotor-Gene Q Real-Time PCR machine (Corbett Research, Australia) according to our previous studies (Askari et al., 2016, 2018c; Askari and Shafiee-Nick, 2019b; Lee and Tansey, 2013; Rahimi et al., 2018). Statistical analysis of gene expression levels was performed by the $2^{-\triangle\triangle Ct}$ method and expressed as fold-changes of the control group.

2.5.5. Nitric oxide and urea assay

The content of the concentration of produced nitrite metabolites was measured using the Griess method to reflect the NO concentration (Ramirez et al., 1996). After treatment of microglia with LPS (1 μg/mL) in the presence or absence of 30, 100, 300 μg/mL the extract, the culture media was collected for NO assay. The culture media (50 μL) was centrifuged at 400 × *g* for 10 min to remove cells and then incubated with equal volumes of Sulfanilamide and N-(1-Naphthyl)-ethylenediamine in 2N hydrochloric acid at controlled room temperature for 10 min. The concentration of nitrite was determined using sodium nitrite standard curve (Askari et al., 2016). Meanwhile, using sodium nitrate as a standard, absorbance values were measured at 540 nm in a spectrophotometer. Urea concentration was also assessed using a colorimetric assay kit from Abcam according to the manufacturer's instruction. In brief, 50 μL of supernatant was used for evaluation of the amount of urea, after that, assay buffer and reaction mix were added, and analyzed in 570 nm with ELISA reader.

2.6. Statistical analysis

The results were presented as means ± SEM (standard deviation). All data were analyzed using one-way analysis of variance (ANOVA) with the Tukey *post hoc* test (for multiple comparisons) to compare means among groups, when passed the normality test Kolmogorov–Simonov. However, data from behavioral test (MWM) were analyzed using repeated measures two-way ANOVA with the Tukey *post hoc* test. A p-value ≤ 0.05, 0.01 and 0.001 was considered statistically significance.

3. Results

3.1. LC-MS analysis of C. spinosa extract

The presence of glycosylated flavonols has been reported in different *C. spinosa* extracts. In total, 25 compounds were identified in the hydro-ethanol extract of the aerial parts of *C. spinosa.* Data concerning the compounds identification are shown in Table 2. The MS spectral data were compared with the reported compounds in some previous literature. Flavonoids were the major identified components and among them, quercetin and kaempferol derivatives were predominate in *C. spinosa,* especially kaempferol, 3-O-rutinoside. These results were in agreement with the literature that reported rutin and kaempferol 3-O-rutinoside as major flavonoids from *C. spinosa* (Fig. 1A–D). Therefore, the aerial parts of *C. spinosa* are good sources of quercetin and kaempferol derivatives for production purposes.

3.2. In vivo studies

3.2.1. The effects of C. spinosa extract and LPS on MWM test

Our result showed that administration of LPS (1 mg/kg/day; i.p.) alone provides higher escape latency time and traveled distance to reach the platform in comparison to the control group in the training phase (days one to five, $p < 0.001$ for both cases, Fig. 2A and C). Furthermore, we observed that there are significant differences in the escape latency time and also traveled distance to reach the platform

Table 2
Peak assignment of metabolites in hydro-ethanol extract of *C. spinosa* using LC–MS in the positive mode.

Compounds	Rt (min)	M^+ (Calc.)	$[M+H]^+$ *m/z*	References
Capparine A	10.1	280.03	281.34	Zhou et al. (2010)
Flazine	4.8	30.08	309.30	Zhou et al. (2010)
Guanosine	4.4	283.09	284.04	Zhou et al. (2010)
Cappariloside A	2.2	334.33	335.34	Yang et al. (2010)
4-hydroxy-1H-indole-3-carboxaldehyde	4.2	161.05	162.96	Zhou et al. (2010)
Kaempferol	4.9	286.05	287.70	Zhou et al. (2010)
Thevetiaflavone	5.1	284.07	285.00	Zhou et al. (2010)
Glucocapparin	2.4	333.02	334.32	Matthäus and Özcan (2002)
Ginkgetin	3.6	566.12	567.18	Zhou et al. (2011)
Sakuranetin	5.2	286.08	286.92	Zhou et al. (2011)
Quercetin-7-rutinoside	3.4	610.15	611.58	Sharaf et al. (1997)
Rutin	9.2	610.15	611.7	Mollica et al. (2017)
Chrysoeriol	4.2	300.06	301.8	Zhou et al. (2010)
Kaempferol hexoside dirhamnoside	2.9	739.22	740.20	Rodrigo et al. (1992)
Kaempferol 3-O-rutinoside	3.6	594.16	595.2	Inocencio et al. (2000)
Kaempferol glucuronide	3.5	462.08	463.44	Bakr and El Bishbishy (2016)
Kaempferol rutinoside hexoside	4	756.21	757.14	Inocencio et al. (2000)
Kaempferol dihexoside dirhamnoside	3.6	90.24	903.54	Bakr and El Bishbishy (2016)
Kaempferol-3-glucoside	3.4	448.10	449.76	Rodrigo et al. (1992)
Quercetin-3-O-hexose-O-pentoside	3.7	596.13	597.36	Bakr and El Bishbishy (2016)
Quercetin dihexoside	3.8	626.15	627.24	Bakr and El Bishbishy (2016)
Quercetin acetyl hexoside	3.7	506.11	507.480	Bakr and El Bishbishy (2016)
Quercetin-3-O-glucoside	3.6	464.10	464.58	Sharaf et al. (2000)
p-Coumaroyl quinic acid	8.3	338.10	339.42	Bakr and El Bishbishy (2016)
Eriodictyol-7-O-rutinoside	3.5	596.17	597.18	Aliyazicioglu et al. (2013)

among 5-days of training in all treated groups ($p < 0.001$ for all cases, Fig. 2A and C). However, the levels of escape latency time and traveled distance to reach the platform were significantly lower in the extract (100 and 300 mg/kg) treatment groups compared to the LPS group ($p < 0.001$ for all cases, Fig. 2A and C). On probe date (6th days), the LPS-treated group significantly spend less time in the target quadrant and cannot recall the platform location in comparison to the control group ($p < 0.001$, Fig. 2B). In contrast, in the probe trial day, the level of spent time in the target quadrant was significantly higher in the groups receiving 100 and 300 mg/kg of the extract in comparison to the LPS-treated group ($p < 0.01$ and $p < 0.001$, respectively, Fig. 2B).

3.2.2. The effects of C. spinosa extract and LPS on brain IL-1β, TNF-α and IL-10 levels

In group received LPS (1 mg/kg/d; i.p), the levels of inflammatory cytokines (IL-1β and TNF-α) were dramatically increased in the whole brain, in comparison to the control group ($p < 0.001$ for both cases, Fig. 3A and B). In contrast, the level of IL-10, anti-inflammatory cytokine, was significantly decreased in the whole brain of LPS-treated groups compared to the control group ($p < 0.001$, Fig. 3C). Interestingly, our findings showed that the groups receiving the dose of 100 and 300 mg/kg of *C. spinosa* extract have lower levels of inflammatory cytokines (IL-1β and TNF-α) in the whole brain homogenate in

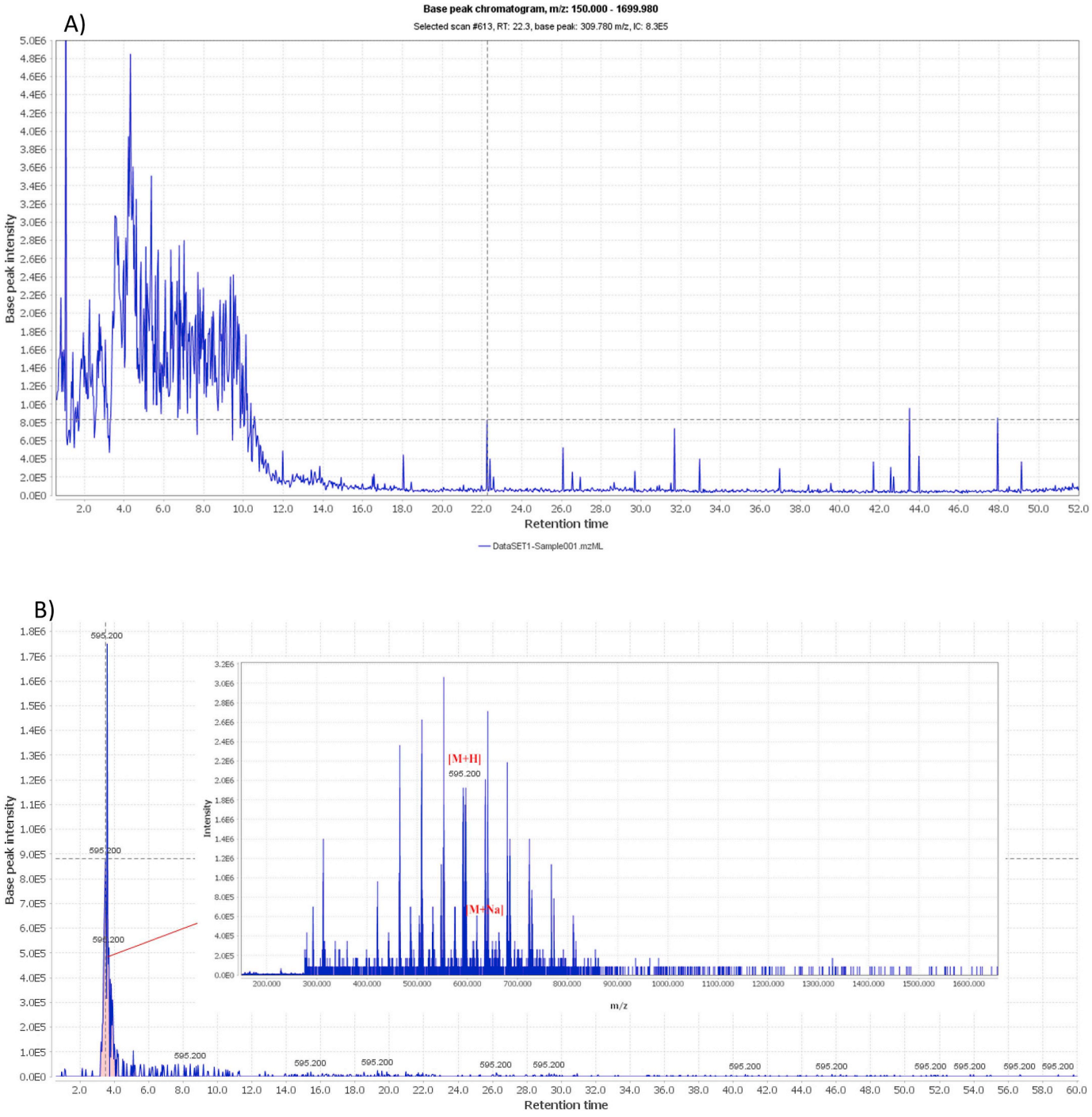


Fig. 1. Chromatogram and corresponding mass adducts. **A)** Total ion chromatogram of C. spinosa L. extract. **B)** Kaempferol 3-O-rutinoside chromatogram and corresponding mass adducts. **C)** Quercetin-3-O-glucoside chromatogram and corresponding mass adducts. **D)** Kaempferol hexoside dirhamnoside chromatogram and corresponding mass adducts.

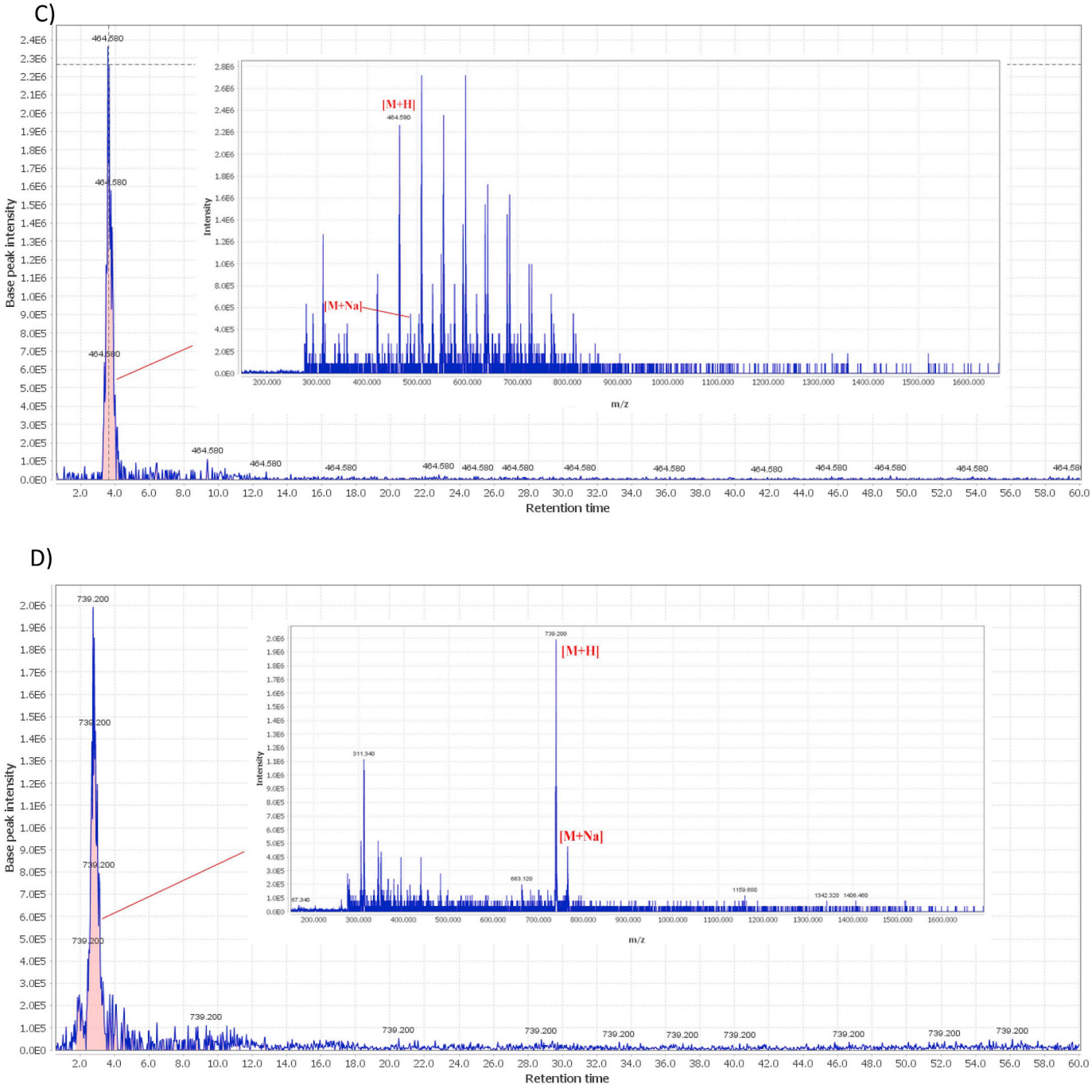


Fig. 1. (*continued*)

comparison to the LPS-treated group ($p < 0.001$–0.01 for all cases, Fig. 3A and B). Moreover, the results indicated that the level of anti-inflammatory cytokine IL-10 was significantly enhanced in the whole brain homogenate of the extract-treated (100 and 300 mg/kg; p.o.) groups, comparing to the LPS-treated group ($p < 0.001$ for both cases, Fig. 3C).

3.2.3. The effects of C. spinosa extract and LPS on brain iNOS and Arg-1 levels, and iNOS/Arg-1 (M1/M2) ratio

The mRNA expression levels of microglial marker iNOS (M1 type) and Arg1 (M2 type) were detected using real time-PCR in the whole brain homogenates. In this experiment, treatment with LPS led to a significant reduction in Arg1 gene expression level in comparison to the control group ($p < 0.01$, Fig. 4A). In contrast, the gene expression level of iNOS was markedly up-regulated in comparison to the control group ($p < 0.001$, Fig. 4B). Accordingly, treatment with LPS significantly propagated the ratio of iNOS/Arg1 (M1/M2) in comparison to the control group ($p < 0.001$, Fig. 4C). On the other hand, in comparison to the LPS group, *C. spinosa* extract (100 and 300 mg/kg) notably increased the level of Arg1 gene expression, while decreased the level of iNOS gene expression ($p < 0.001$ for all cases, Fig. 4A and B). Moreover, the extract (100 and 300 mg/kg) significantly diminished the ratio of iNOS/Arg1 (M1/M2), compared to the LPS-treated group ($p < 0.001$ for both cases, Fig. 4C).

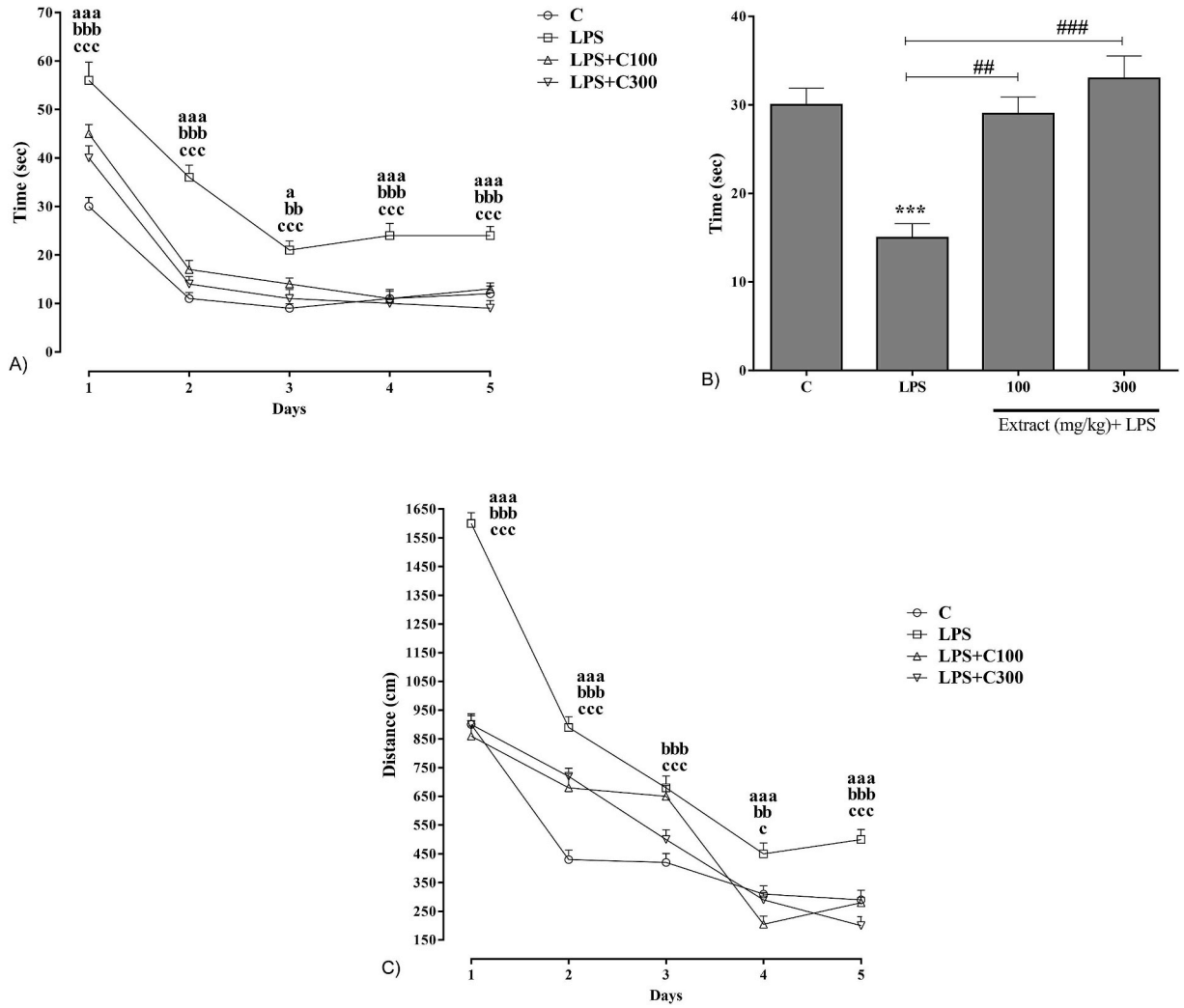


Fig. 2. The effects of *C. spinosa* extract and LPS on the levels of time latency (A), time spent in target quadrant (B) and distances to reach the platform (C), in MWM test. The data were presented as mean ± SEM; n = 10 per group. [a] $p < 0.05$ and [aaa] $p < 0.001$, compared dose of 100 mg/kg to LPS group; [bb] $p < 0.01$ and [bbb] $p < 0.001$, compared dose of 300 mg/kg to LPS group; [c] $p < 0.05$ and [ccc] $p < 0.001$, compared control group to LPS group. ##$p < 0.01$ and ###$p < 0.001$, compared with the LPS group; ***$p < 0.001$, compared with control group. The presented data in Fig. 2A) and C) were analyzed using repeated measure two-ANOVA with the Tukey *post hoc* test. However, The presented data in Fig. 2B) were analyzed by one-way ANOVA with the Tukey *post hoc* test. LPS, lipopolysaccharide; SEM, standard error mean.

3.3. In vitro studies

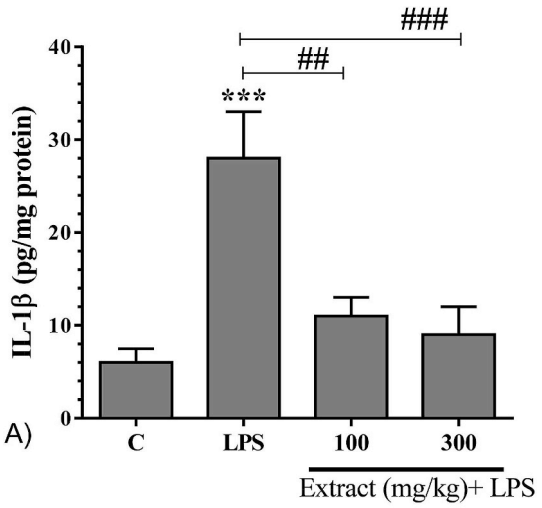
3.3.1. The effects of C. spinosa extract and LPS on the proliferation of microglial cells

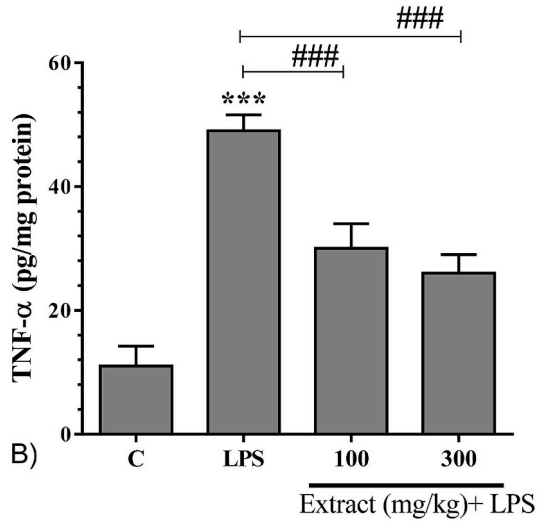
C. spinosa (1–300 μg/mL) and dexamethasone (0.5 μM) exerted no significant effects on proliferation level of microglial cells (Fig. 5A). Our records indicated that LPS (1 μg/mL) significantly reduced the level of cell proliferation compared to the respected control group ($p < 0.001$, Fig. 5B). The extract (100 and 300 μg/mL) and also dexamethasone (0.5 μM) significantly enhanced the cell proliferation of LPS-treated microglial cells compared to the LPS group ($p < 0.05$–0.001 for both cases, Fig. 5B).

3.3.2. The effects of C. spinosa extract and LPS on the pro-inflammatory cytokine level

As presented in Fig. 6, treatment of the cells with LPS significantly increased the mRNA expression levels of pro-inflammatory cytokines (TNF-α, IL-6, and IL-1β) when compared to the non-stimulated cells ($p < 0.001$, Fig. 6A-C). In the presence of LPS-stimulation, the mRNA expression levels of TNF-α ($p < 0.01$–0.001 for all cases), IL-1β ($p < 0.01$–0.001 for all cases), and IL-6 ($p < 0.001$ for all cases) were markedly decreased following the treatment with *C. spinosa* extract (30–300 μg/ml, in a concentration dependent manner) or dexamethasone (0.5 μM) (Fig. 6A–C). In our study, we also assessed the protein levels of pro-inflammatory cytokines (TNF-α, IL-6, and IL-1β) using ELISA method. In the presence of LPS, the protein levels of these pro-inflammatory cytokines were notably elevated in comparison to the non-stimulated cells ($p < 0.001$ for all cases, Fig. 7A). In contrast, treatment with the extract (30–300 μg/ml, in a concentration-dependent manner) or dexamethasone (0.5 μM) resulted in a significant decrease in the levels of TNF-α ($p < 0.01$–0.001 for all cases), IL-6 ($p < 0.001$ for all cases), and IL-1β ($p < 0.01$–0.001 for all cases) when compared to the LPS-stimulated cells (Fig. 7A–C).

We also revealed that in the extract treated groups the levels of TNF-α (30 and 100 μg/ml of the extract; 0.001 for all cases; Figs. 6A and 7A), IL-6 (30 and 100 μg/ml of the extract; 0.001 for all cases; Figs. 6B, and Fig. 7B) and IL-1β (30, 100 and 300 μg/ml of the extract; $p < 0.01$–0.001 for all cases; Figs. 6C, and Fig. 7C) cytokines at both gene expression and protein levels were remarkably lower than dexamethasone (0.5 μM; Figs. 6 and 7) treated group.





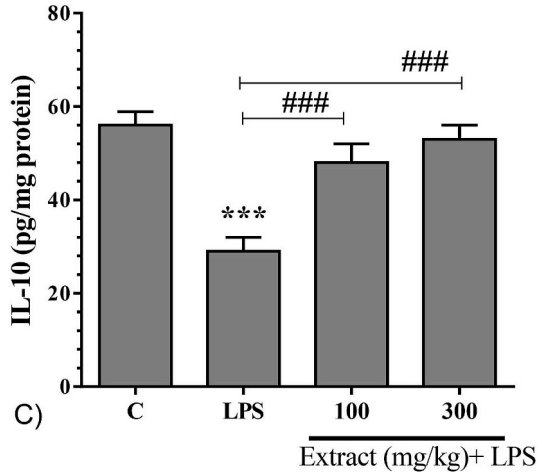
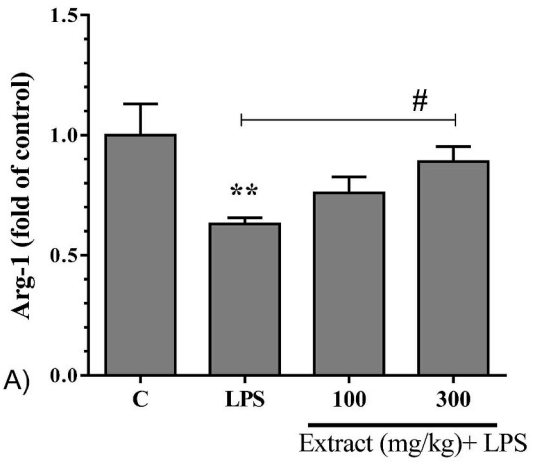
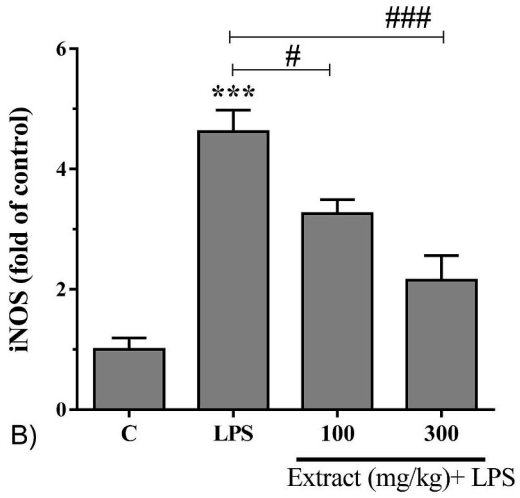


Fig. 3. Effects of *C. spinosa* extract on pro-inflammatory factors of **A)** TNF-α, **B)** IL-6, and **C)** IL-1β in the whole brain of rats. Concentrations of TNF-α, IL-6, IL-1β were determined using ELISA. Data represented as mean ± SEM; n = 10 per group. ##p < 0.01 and ###p < 0.001, compared with LPS group; ***p < 0.001, compared with control group. The presented data were analyzed by one-way ANOVA with the Tukey *post hoc* test. LPS, lipopolysaccharide; SEM, standard error mean; TNF-α, Tumor necrosis factor-α, IL-1β, Interleukin 1-β; IL-6, Interleukin-6.





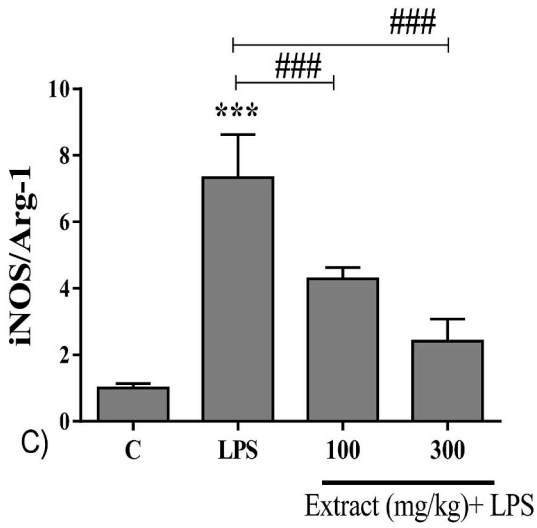


Fig. 4. Effects of *C. spinosa* extract on the mRNA expression levels of iNOS **(A)** and Arg1 **(B)**, and iNOS/Arg1 ratio **(C)** in the whole brain of rats. The expression levels of iNOS and Arg1 were evaluated by real time-PCR. Data represented as mean ± SEM; n = 10 per group. ###p < 0.001, compared with LPS group; ***p < 0.001, compared with the control group. The presented data were analyzed by one-way ANOVA with the Tukey *post hoc* test. LPS, lipopolysaccharide; SEM, standard error mean; Arg1, arginase-1; iNOS, inducible nitric oxide synthase.

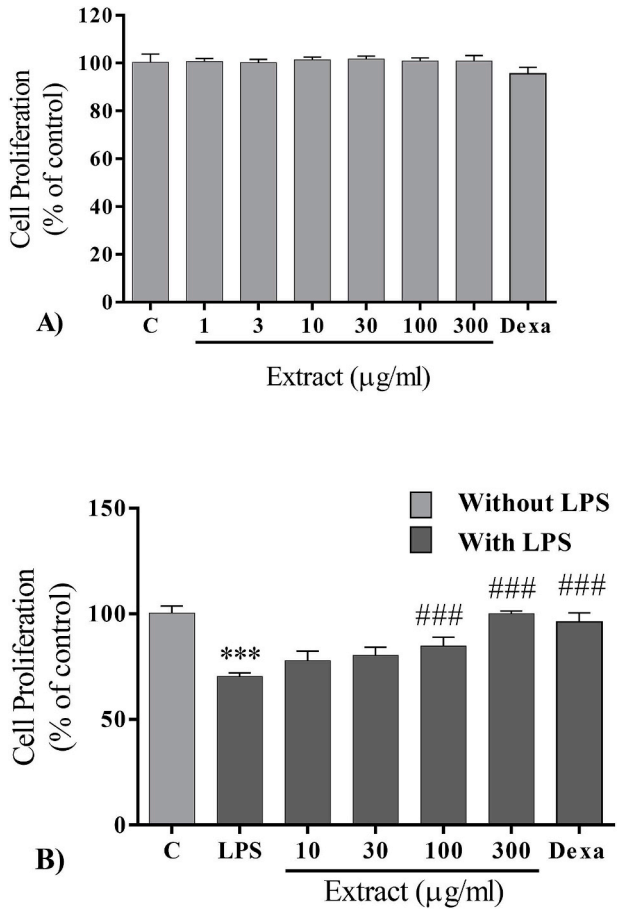


Fig. 5. The effects of *C. spinose* extract on proliferation level of microglia cells. **(A)** Cells were treated with the extract (1–300 µg/mL) for 48 h. **(B)** Cells were treated with the extract for 24 h before exposure to LPS (1 µg/mL). Cell proliferation was measured using MTT assay. Data represented as mean ± SEM; n = 6 per group. ###p < 0.001, compared with LPS group; ***p < 0.001, compared with the control group. The presented data were analyzed by one-way ANOVA with the Tukey *post hoc* test; LPS, lipopolysaccharide; SEM, standard error mean; Dexa, dexamethasone (0.5 µM).

3.3.3. The effects of C. spinosa extract and LPS on the level of PGE_2 and COX-2

LPS stimulation caused a significant increase in the production level of PGE_2 in comparison to the control group ($p < 0.001$, Fig. 8A). In contrast, a significant inhibition of PGE_2 production was found with either all evaluated concentrations of the extract (30–300 µg/ml) or dexamethasone (0.5 µM) compared to the LPS group ($p < 0.001$ for all cases, Fig. 8A). In order to determine whether the inhibitory effects of *C. spinosa* extract on PGE_2 production were possibly due to the decrease in the mRNA expression level of COX-2, an enzyme responsible for PGE_2 production, the mRNA expression level of COX-2 was also examined by real time-PCR analysis. In response to LPS, the gene expression of COX-2 was markedly up-regulated comparing to the non-stimulated cells ($p < 0.001$, Fig. 8B). However, in comparison to the LPS-treated group, treatment with *C. spinosa* extract (30–300 µg/ml, concentration-dependently) or dexamethasone (0.5 µM) could significantly attenuate the gene expression level of COX-2 ($p < 0.001$–0.01 for all cases, Fig. 8B). Moreover, the impact of dexamethasone (0.5 µM) on the reduction of PGE_2 level was significantly greater than all assessed concentrations (30–300 µg/ml) of C. *spinosa* extract ($p < 0.001$ to 0.01 for all cases, Fig. 8A), however, COX-2 expression level was observed significantly higher only at 30 µg/ml concentration of the extract in comparison to the dexamethasone (0.5 µM) ($p < 0.001$, Fig. 8B).

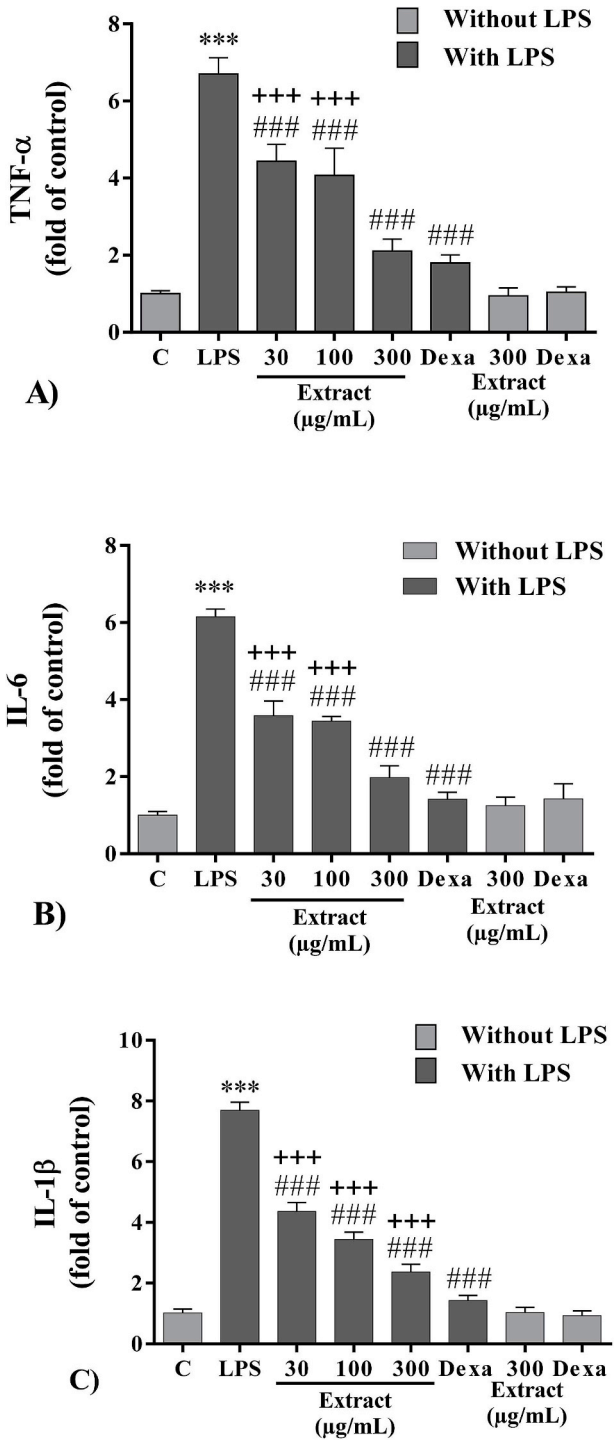


Fig. 6. Effects of *C. spinose* extract on the mRNA expression levels of pro-inflammatory factors of **A)** TNF-α, **B)** IL-6, and **C)** IL-1β in LPS-stimulated microglial cells. The levels of TNF-α, IL-6, and IL-1β were assessed using real-time PCR. Data represented as mean ± SEM; n = 6 per group. ###p < 0.001, compared with LPS group; ***p < 0.001, compared with the control group; + + + p < 0.001, compared with Dexa group. The presented data were analyzed by one-way ANOVA with the Tukey *post hoc* test. LPS, lipopolysaccharide; SEM, standard error mean; TNF-α, Tumor necrosis factor-α, IL-1β, Interleukin 1-β; IL-6, Interleukin-6; Dexa, dexamethasone (0.5 µM).

3.3.4. The effects of C. spinosa extract and LPS on the levels of iNOS, Arg-1 and iNOS/Arg-1(M1/M2) ratio

To further investigate the effect of *C. spinosa* on microglia activation and function, the mRNA expression of microglial marker iNOS (M1

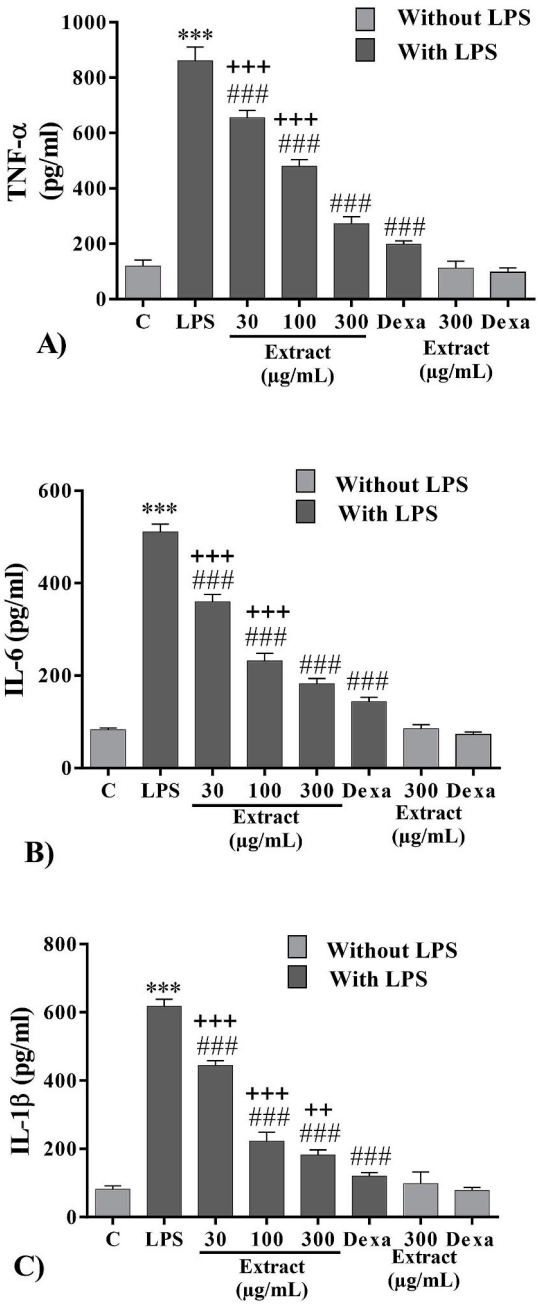


Fig. 7. Effects of *C. spinosa* extract on pro-inflammatory factors **A)** TNF-α, **B)** IL-6, and **C)** IL-1β in LPS-stimulated microglial cells. Concentrations of TNF-α, IL-6, IL-1β were determined using ELISA. Data represented as mean ± SEM; n = 6 per group. ###p < 0.001, compared with LPS group; ***p < 0.001, compared with control group; ++ p < 0.01 and +++ p < 0.001, compared with Dexa group. The presented data were analyzed by one-way ANOVA with the Tukey *post hoc* test. LPS, lipopolysaccharide; SEM, standard error mean; TNF-α, Tumor necrosis factor-α, IL-1β, Interleukin 1-β; IL-6, Interleukin-6; Dexa, dexamethasone (0.5 μM).

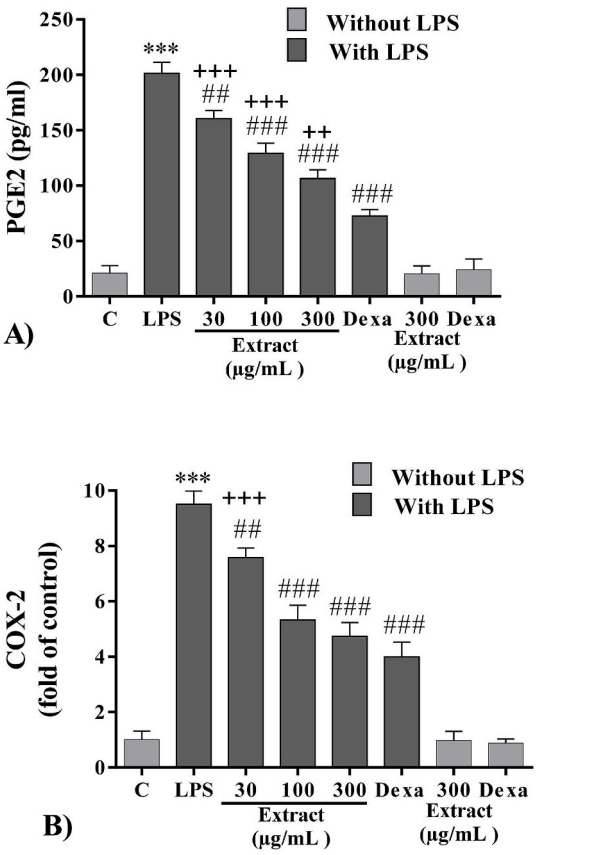


Fig. 8. Effects of *C. spinose* extract on LPS-induced PGE_2 production **(A)** and COX-2 mRNA expression **(B)**. The levels of PGE_2 and COX-2 were evaluated by ELISA and real time-PCR, respectively. Data represented as mean ± SEM; n = 6 per group. ##P < 0.01, ###p < 0.001, compared with LPS group; ***p < 0.001, compared with control group; ++ p < 0.01 and +++ p < 0.001, compared with Dexa group. The presented data were analyzed by one-way ANOVA with the Tukey *post hoc* test. LPS, lipopolysaccharide; SEM, standard error mean; COX-2, cyclooxygenase-2, PGE_2, Prostaglandin E_2, Dexa, dexamethasone (0.5 μM).

cells) and Arg1 (M2 cells) were detected using the real time-PCR. In the presence of LPS-stimulation, the gene expression level of iNOS was markedly up-regulated in comparison to the non-stimulated cells (p < 0.001, Fig. 9A). In this experiment, LPS also led to a reduction in Arg1 gene expression level, but this was not statistically significant in comparison to the control group (Fig. 9B). Collectively, our analysis showed that LPS-stimulation significantly enhances iNOS/Arg1 (M1/M2) ratio compared to the control cells (p < 0.001, Fig. 9C).

In comparison to the LPS group, treatment with either *C. spinosa* extract (30–300 μg/ml; in a concentration-dependent manner) or dexamethasone (0.5 μM) could up-regulate the level of Arg1 gene expression (p < 0.001 for all cases, Fig. 9B), reduced iNOS gene expression level, (p < 0.001 for all cases, Fig. 9A), and finally attenuate the level of iNOS/Arg1 ratio (M1/M2; p < 0.001 for all cases, Fig. 9C), compared to the LPS-treated cells. Moreover, the impact of dexamethasone (0.5 μM) reduced iNOS gene expression greater than those group treated with C. *spinosa* extract (30–300 μg/ml) (p < 0.001 for all cases, Fig. 9A). At 30 and 100 μg/ml concentration of the extract, the levels of Arg1 gene expression (p < 0.001 for both cases, Fig. 9B) and iNOS/Arg1 ratio (M1/M2; p < 0.001 for both cases, Fig. 9C) were observed significantly lower and greater than that group treated with dexamethasone (0.5 μM).

3.3.5. The effects of C. spinosa extract and LPS on the levels of NO, urea and NO/urea (M1/M2) ratio

Following the LPS stimulation, the levels of NO production (p < 0.001, Fig. 10A) and urea production (p < 0.001, Fig. 10B) were significantly increased and deceased, respectively, compared to the control group. LPS stimulation led to a marked increment in the level of NO/urea ratio compared to the control group (p < 0.001, Fig. 10C). In comparison to the LPS group, all concentrations of the extract (30–300 μg/ml; in a concentration-dependent manner) and also dexamethasone (0.5 μM) significantly inhibited NO production (p < 0.001 for all cases, Fig. 10A), and meaningfully increased the

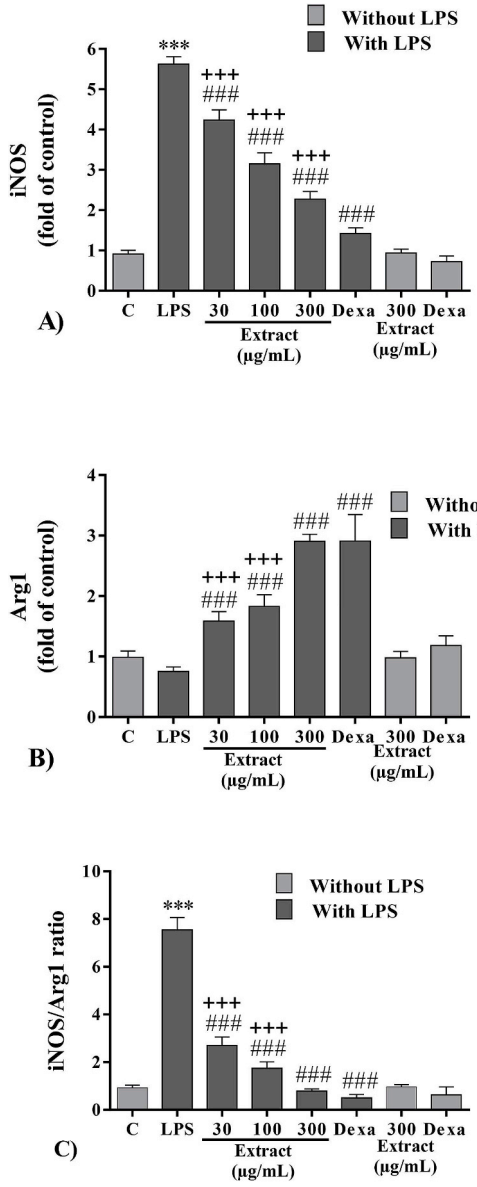


Fig. 9. Effects of *C. spinosa* extract on the mRNA expression levels iNOS **(A)** and Arg1 **(B)**, and iNOS/Arg1 ratio **(C)** in LPS-stimulated microglial cells. The expression levels of iNOS and Arg1 were evaluated by real time-PCR. Data represented as mean ± SEM; n = 6 per group. ###p < 0.001, compared with LPS group; ***p < 0.001, compared with control group; +++ p < 0.001, compared with Dexa group. The presented data were analyzed by one-way ANOVA with the Tukey *post hoc* test. LPS, lipopolysaccharide; SEM, standard error mean; Arg1, arginase-1; iNOS, inducible nitric oxide synthase.

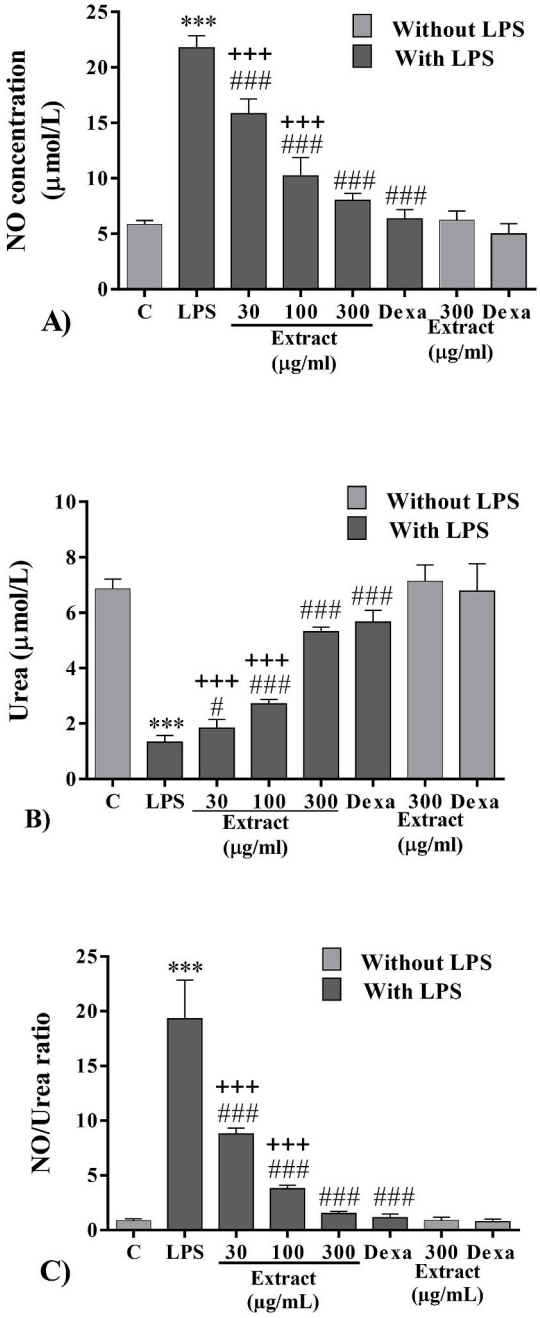


Fig. 10. Effects of *C. spinosa* extract on LPS-induced NO **(A)**, urea **(B)** production, and NO/urea (M1/M2) ratio **(C)**. Concentrations of NO and Urea were determined using ELISA. Data represented as mean ± SEM; n = 6 per group. ###p < 0.001, compared with LPS group; ***p < 0.001, compared with control group; +++ p < 0.001, compared with Dexa group. The presented data were analyzed by one-way ANOVA with the Tukey *post hoc* test. LPS, lipopolysaccharide; SEM, standard error mean; NO, nitric oxide metabolites; Dexa, dexamethasone (0.5 µM).

level of urea ($p < 0.05$–0.001 for all cases, Fig. 10B) in comparison to the LPS group. Eventually, in both the extract (30–300 µg/ml; in a concentration-dependent manner) and dexamethasone (0.5 µM) treated groups, the levels of NO/urea ratio were notably decreased in comparison to the LPS group ($p < 0.001$ for all cases, Fig. 10C). We observed that the effects of dexamethasone (0.5 µM) on the reduction of NO production ($p < 0.001$ for both cases, Fig. 10A) and also NO/urea ratio ($p < 0.001$ for both cases, Fig. 10C), and on the increment of urea production ($p < 0.001$ for both cases, Fig. 10B) were more powerful than those LPS group treated with C. *spinosa* extract (30 and 100 µg/ml).

4. Discussion

To the best of our knowledge, this is the first study reporting the protective effects of *C. spinosa* extract on the reduction of LPS-induced systemic inflammation associated with cognitive impairment and neuro-inflammation. In the present study, we assessed the protective

effects of *C. spinosa* against LPS-induced inflammation both *in-vivo* and *in-vitro* models. Briefly, our results showed that *C. spinosa* significantly and dose-dependently reduced the level of inflammation, and the spatial memory dysfunction following the LPS-induced systemic inflammation.

Several studies indicated that inflammation plays an essential role in memory dysfunction. Contextually, it has been reported that systemic inflammation due to LPS, leads to increasing the activities of microglia and astrocytes in the brain, which they subsequently result in spatial memory impairments (Labouesse et al., 2015; Valero et al., 2014). Neuro-inflammation following the chronic activation of microglia contributes to neurodegenerative processes. In this regard, several studies have shown that controlling the pro-inflammatory responses by microglial cells are proposed as a promising therapeutic strategy for neurodegenerative disease (Baune, 2015; Wang et al., 2015).

In the current study, we observed that systemic and long-term administration of LPS leads to both spatial memory impairments (using MWM test) and neuro inflammation. In fact, LPS-treated animals significantly showed longer time spent and traveled distance to find the platform than the control group. Moreover, following the platform removed, the time spent in target quadrant was lower in animals received LPS than the control group. These findings imply that the spatial memory has been impaired in LPS treated animal, because they cannot remember the location of platform. Additionally, we found that systemic administration of LPS notably increases the levels of inflammatory cytokines including IL-1β and TNF-α, and significantly decreases the level of anti-inflammatory cytokine IL-10, in comparison to the control group. Interestingly, in the LPS-treated group, we found that the levels of iNOS (namely M1 marker) and Arg-1 (namely M2 marker) were significantly increased and decreased, respectively, which leads to an increase in the level of iNOS/Arg-1 (M1/M2) ratios towards M1 inflammatory phenotypes, in comparison to the control group. In agreement with these results, previous studies also showed that systemic administration of LPS impairs spatial memory assessing by MWM and Y-maze tests (Beheshti et al., 2019; Labouesse et al., 2015; Valero et al., 2014; Yamada et al., 1999). Furthermore, it has been reported that the systemic inflammation by LPS leads to the activation of glial cells and production of high levels of inflammatory markers (IL-1β, IL-6 and, TNF-α) which potentially are associated with neuronal dysfunction, especially in the hippocampus region, and memory and learning impairments (Beheshti et al., 2019; Labouesse et al., 2015; Valero et al., 2014; Yamada et al., 1999).

In the LPS-induced inflammation model, activated microglia upregulate the expression of iNOS and resultant NO (Bai et al., 2015). Upregulation of iNOS is considered an important oxidative component of the microglial inflammatory response to toxins and noxious stimuli in the central nervous system (VL et al., 1994; Wong et al., 1996). Excess NO production by iNOS causes neuronal injury and death (Aquilano et al., 2008). LPS-mediated neurotoxicity may be partly attributed to oxidative stress. By *in vitro* evaluation, our results showed that primary microglia, under the LPS challenge, produced NO, TNF-??, IL-1β, and IL-6 and PGE_2 involved in process of inflammation. Indeed, LPS could polarize microglia into the M1 pro-inflammatory phenotype and also reduced the expression of M2 anti-inflammatory marker which led to inflammatory responses. These results were consistent with the previous findings (Askari et al., 2018b; Yang et al., 2017).

In the present study, the anti-neuroinflammatory and microglia-modulating effects of *C. spinosa* were evaluated using both *in vivo* and *in vitro* models of inflammatory responses in rats and microglial cells, respectively. As results, we found that *C. spinosa* attenuated LPS-induced spatial memory impairment in MWM test. In this regard, several studies support our findings related to the neuro-protection of *C. spinosa*. In amyloid-beta (Aβ) peptide injected rat model of AD, *C. spinosa* down-regulated the genes involved in inflammation (Mohebali et al., 2018). The protective properties of the plant against neuroinflammation was reported due to its antioxidant effects and the presence of flavonoids including rutin (Mohebali et al., 2018). In line with our results, Turgut and coworkers showed that 100 and 200 mg/kg of *C. spinosa* improve cognitive impairments by D-galactose (Turgut et al., 2015). In another study, Goel et al. demonstrated that *C. spinosa* enhanced spatial memory in LPS-induced model of cognitive impairments (Goel et al., 2016). Although our results in consistent with this study, but there are major differences between our and their model. In this regard, Goel and their colleagues performed their study with lower dose (175 μg/kg) and time of exposure (7 days) to LPS, but in the current study, we carried out the study with optimal dose of LPS (1 mg/kg, described elsewhere) and longer duration of exposure (33 days). Additionally, in our experiment, we examined the levels of inflammatory (IL-1β and TNF-α) and anti-inflammatory (IL-10) cytokines as well as the levels of iNOS, Arg-1 and iNOS/Arg-1 ratio, which have been not evaluated in Goel study. Intriguingly, in the extract treated group, we observed that the levels of inflammatory (IL-1β and TNF-α) cytokines, iNOS and iNOS/Arg-1 ratio were dramatically lower than the LPS-treated group, while anti-inflammatory cytokine (IL-10) was more than the LPS-treated group. Moreover, this is the first observation to demonstrate that *C. spinosa* is capable to switch microglia polarization from a pro-inflammatory M1 state towards anti-inflammatory state M2.

Experimentally, our data showed that *C. spinosa* concentration-dependently suppressed production of NO, and inflammatory mediators produced by LPS-stimulated microglial cells. These inhibitory effects were mediated by decreasing both mRNA expression and protein levels of M1 phenotype markers and pro-inflammatory cytokines, including TNF-α, IL-1β, IL-6, iNOS and COX-2. Additionally, in the present study, we realized that *C. spinosa* reduces Arg1 expression and increased resultant product, urea. It was associated with the switch of microglia polarization from pro-inflammatory M1 to anti-inflammatory M2 type indicated by increased Arg1/iNOS ratio as well as reduced NO/urea ratio. However, further investigation should be considered to support our data regarding the polarization of M1/M2 cells by immunocytochemistry and flow cytometey techniques. The results were consistent with previous studies reported the anti-inflammatory properties of *C. spinosa* (Arena et al., 2008; Hamuti et al., 2017; Kwak et al., 2002; Lee et al., 2005). The anti-inflammatory activity of ethanolic extracts of *C. spinosa* has been demonstrated in the previous experiments. The extracts up-regulated the expression of a cluster of differentiation (CD) 40, CD 80 and CD 86 and also counteracted the release of pro-inflammatory cytokines including IL-1β, IL-12 p40, IL-6 and IFN-γ induced by LPS in dendritic cells (Hamuti et al., 2017). In another study, Isoginkgetin, and ginkgetin, and other bioflavonoids isolated from *C. spinosa* prevented NF-κB activation (Zhou et al., 2011). Anti-inflammatory effects of ginkgetin have been suggested to be mediated through suppression of COX-2 expression in LPS- treated macrophages *in vitro* and also COX-2 expression and PGE_2 production in mouse skin treated with the 3d treatment of 12-O-tetradecanoyl phorbol-13-acetate (TPA) (Kwak et al., 2002). *In vitro* exposure of human peripheral blood mononuclear cells to *C. spinosa* methanolic extract inhibited replication of HSV-2, and production of IFN- γ, TNF- α and IL-12 in peripheral blood mononuclear cells infected with HSV-2. Its polyphenolic active components, in particular flavonoids, are also found to possess antiviral and immunomodulatory properties (Arena et al., 2008).

As our study limitation, Apart from measurement of cytokine expression level in the whole brain, histological analysis seems necessary to show the degeneration of neurons and to confirm the establishment of neuroinflammation and cognitive impairment model, but unfortunately we performed the study and measured the cytokine expression levels in the whole brain and lost the brain for histopathological evaluation. Therefore, we can suggest this important issue for investigations in further studies and researchers.

In conclusion, we showed that treatment with *C. spinosa* enhances memory and cognitive behavior and anti-inflammatory cytokine IL-10 following the LPS-induced neuro-inflammation. Additionally, we found that *C. spinosa* possibly exerts its effect through modulation of microglia

polarization by shifting inflammatory phenotype (M1) towards anti-inflammatory phenotype (M2) cells. Our finding provides evidence that *C. spinosa* has neuroprotective effect, and might be considered as an effective therapeutic agent for the treatment of neurodegenerative diseases that are accompanied by inflammation, such as AD. However, further investigations seem necessary to clarify the mechanism underlying the anti-inflammatory effects of *C. spinosa* and determine its most biologically active chemical constituents.

Declaration of transparency and scientific rigor

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigor of preclinical research recommended by funding agencies, publishers and other organizations engaged with supporting research.

Author contributions statement

VA, HRK and VB contributed conception and design of the study; VA, HRA, VB, EMV, HRM and AR performed the study; VA, VB, MI and AR performed the statistical analysis; AR, VB and HRK wrote the first draft of the manuscript; VB, VA, AR, EMV, HRM, MM, MI and HRK wrote sections of the manuscript; VA and VB provides final revision of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

Declaration of competing interest

The authors declare no conflicts of interest.

Acknowledgments

This study financially supported by the research council of Mashhad University of Medical Sciences (961339, IR.MUMS.MEDICAL.REC.1397.041).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2020.112706.

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